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No. 3 is the correct journal as cited from Jrnl. of Mass spectrometry, vol. 33, 264-273 (1998).

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**From:** Wessendorf,Teresa
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Please forward:
1. Proceedings of the 45th ASMS conference onMass spectrometry and allied Topics, Palm Springs, June 1-5, 1997, p. 907, Siegel et al
2. Proceedings of the 44th ASMS conference on mass spectrometery and allied topics, Portland, Or. May 12-16, 1996, p. 1424, Siegel et al.
3. Protein Science, 3, 81, (1994), Hutchens et al
4. Rapid Commun. Mass Spectrom. 7, 576 (1993).
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# A Rapid Method for Screening Low Molecular Weight Compounds Non-Covalently Bound to Proteins Using Size Exclusion and Mass Spectrometry Applied to Inhibitors of Human Cytomegalovirus Protease

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## INTRODUCTION

A property of a useful drug candidate is the ability to form a tightly bound non-covalent complex with its target protein. Using the model system of human cytomegalovirus protease (CMVP), a simple, reliable and rapid method was developed for identifying low molecular weight inhibitors of CMVP which bind non-covalently to the enzyme. The technique utilizes size exclusion GPC spin columns and/or ultrafiltration devices (microconcentrators) for isolating non-covalently bound inhibitor-protease complexes prepared under native conditions, which are then introduced under denaturing conditions into an ESI mass spectrometer, for monitoring and quantitating the individual components of inhibitor and protease. The sample preparation, isolation and detection steps are performed and optimized individually. The methodology is simple to apply and rapid to implement, and allows the characterization of specific and non-specific binding of low molecular weight molecules to protease and the quantitation of the molar ratio of inhibitor to protease in the complex.

## EXPERIMENTAL METHOD

Proteases: Wild type CMVP (MW 28,040.6) and mutants A144L (MW 28,082.8), A144D/C87A/C138A/C161A (MW 27,956.7), S132A (MW 28,024.6) and E122V/A144G (MW 27,996.6) were used in the studies. Inhibitors: The inhibitors of CMVP used in these studies are a peptidic difluoroketone, DFK (MW 988.5) (1), two peptidic trifluoromethylketones, TFMK-1 (MW 545) (2) and TFMK-2 (MW 465) (3), and a dibromo quinazoline, DBQ (MW 489) (4). Sample Preparation: CMVP (60 μM) was incubated with a known molar excess of inhibitors in 10mM ammonium acetate (pH 7.5) for 1 hr at 37 °C. The samples were then assayed by size exclusion methods. Gel Permeation Chromatography (GPC) (Spin Column): GPC spin columns were prepared by filling 1 mL disposable polypropylene syringes (3 mm i.d.) with Sephadex G-25 resin (Pharmacia). The column was centrifuged at 900 x g and the filtrate analyzed. The resin traps molecules <3,000 Da and elutes proteins. Ultrafiltration: Ultrafiltration microconcentrators (3,000 Da cut-off, Amicon Microcon-3, Beverly, MA) were centrifuged at 14,000 x g for 10 minutes. After centrifugation, the filtrate contains material <3,000 Da and the retentate contains material >3,000 Da, such as CMVP or CMVP bound to inhibitor. Mass Spectrometry: Electrospray ionization mass spectra were obtained with a Micromass Quattro triple quadrupole mass spectrometer equipped with a Micromass electrospray source, rf hexapole lens and Megaflow gas nebulizer probe.

## RESULTS and DISCUSSION

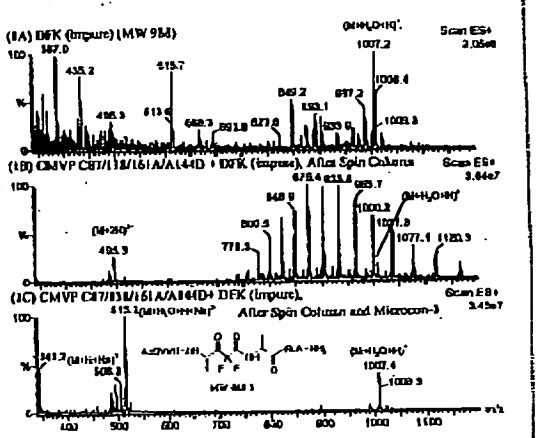
1) Rapid Screening Size Exclusion-Mass Spectral Assay for Non-Covalently Bound Complexes

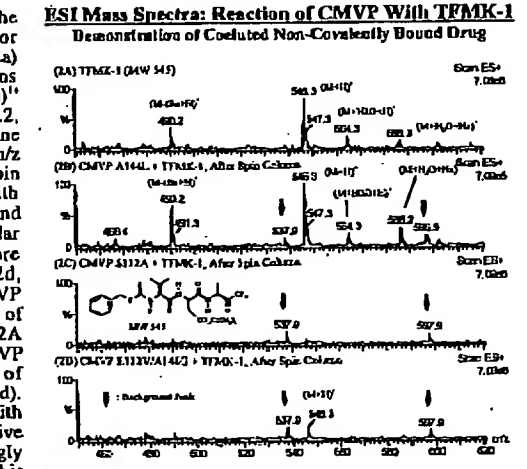
An impure sample of DFK (MW 988.5) (1) (see ESI mass spectrum Figure 1a) was incubated with CMVP A144D/C87A/C138A/C161A in a molar ratio of CMVP:DFK of 1:~10. The resulting mixture was transferred to a GPC spin column and the eluate was analyzed by ESI/MS. As illustrated in Figure 1b, the ESI mass spectrum of the eluate consists of a series of multiply charged peaks related to CMVP in the m/z region of 700-1200 and a series of peaks related to DFK (1) at m/z 1007.4, 495.3 and 486.7 corresponding to $(M+H_2O+H)^{1+}$, $(M+2H)^{2+}$ and $(M+2H-H_2O)^{2+}$, respectively. Note that components corresponding to (1) and the hydrated form of (1) eluted from the spin column together with CMVP demonstrating non-covalent binding of the compounds to CMVP, otherwise, only CMVP would have eluted from the spin column. As a control, DFK (1) alone, at the same concentration used in the incubation experiment, was passed through the spin column, and all peaks corresponding to DFK (1) were absent. Note also that all the minor impurities present in the original DFK (1) sample (Figure 1a) are absent (Figure 1b), indicating that they did not specifically bind to CMVP. Thus, this method for characterizing non-covalent binding is applicable for the analysis of mixtures of compounds; non-covalently bound inhibitors will be selectively coeluted with CMVP while other unbound low molecular weight components will be trapped by the GPC spin column resin. (Similar results were obtained when using a microconcentrator and analyzing by ESI/MS the retentate of the incubated mixture.)

The spin column eluate was next placed into a microconcentrator with a denaturing solution of 3% acetic acid in 1:1 acetonitrile:water. The filtrate was collected and analyzed by ESI/MS (Figure 1c). Note the absence in the mass spectrum of the ion distribution corresponding to the CMVP and the presence of singly, doubly and triply charged peaks corresponding to (1) and the hydrated form of (1).

2) Specificity of Non-Covalently Bound Complexes

To examine specifity of binding of compounds to CMVP, two enzymatically inactive mutants of the protease were used. CMVP S132A contains alanine substituted for serine at amino acid 132; this serine is the active site nucleophile which plays a key role in catalysis by CMVP and is expected to be essential for CMVP to bind tightly at the carbonyl carbon of TFMK-1. CMVP E122V lacks the glutamic acid residue which forms a salt bridge in the wild type CMVP; this mutation probably disrupts the normal conformation of the protease. The ESI mass spectrum for inhibitor TFMK-1 (MW 545) (2) (Figure 2a) exhibits the characteristic molecular ions $(M+H)^{1+}$, $(M+H_2O+H)^{1+}$, $(M+H_2O+Na)^{1+}$ and $(M+H_2O+K)^{1+}$ at m/z 546.2, 564.2, 586.2 and 602.1, respectively, as well one fragment ion $(M-C(CH_3)_3+2H)^{1+}$ at m/z 490.1. The ESI mass spectra of the spin column eluates of TFMK-1 incubated with CMVP's A144L (wild type), S132A and E122V/A144G, each prepared at a molar ratio of CMVP:TFMK-1 of 1:40, are illustrated in Figures 2b, 2c and 2d, respectively. TFMK-1 coelutes with CMVP A144L (in a CMVP:TFMK-1 molar ratio of 1:1), does not coelute with CMVP S132A and essentially does not coelute with CMVP E122V/A144G (a molar ratio of CMVP:TFMK-1 of 1:<0.05 was recovered). These coelution results are consistent with the requirement of enzymatically active protease for binding to TFMK-1, strongly suggesting that the binding of this compound to CMVP is specific.




3) Competition Study of Inhibitor Mixture with CMVP

A mixture of CMVP A144L with TFMK-1 (MW 545) (2), TFMK-2 (MW 465) (3) and DBQ (MW 489) (4), was prepared with molar ratios of 1:5:5:5, respectively. The ESI mass spectrum exhibited peaks with corresponding molar ratios of 1 : 0.15 : 0.083 : 2.17. These results indicate that under the experimental conditions the tightest binding compound to the protease relative to that of the GPC packing material was DBQ.